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Apoptotic Effects of Heparin on Lymphoblasts, Neutrophils, and Mononuclear Cells: Results of a Preliminary In Vitro Study

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In this study the apoptotic effects of heparin on lymphoblasts, neutrophils, and mononuclear cells were evaluated by flow cytometry for detection of sub-G_1 peak, in vitro. Ten children with acute lymphoblastic leukemia (ALL) at diagnosis (Group I), six children with ALL at relapse (Group II), and 10 healthy children (controls) were included in this study. Lymphoblasts in ALL patients, and neutrophils and mononuclear cells in controls, were incubated in increasing heparin concentrations (0, 5, 10, 20 U/ml). Flow cytometric analyses were performed at 0, 1, and 2 hours of incubation in heparin for determination of the apoptotic effects of heparin. In Group I apoptosis was detected in all different levels of heparin concentration except 0 U/ml at 0, 1, and 2 hours. The apoptotic effects of heparin on blast cells peaked at the first hour in 5-, 10-, and 20-U/ml heparin concentrations ($p < 0.0001$). In Group II similar findings were observed only at zero hour and apoptosis was higher than those in Group I except in 5-U/ml heparin concentration ($p < 0.001$). Apoptosis was found to increase with heparin levels in both groups ($p < 0.02$). In the control group, apoptosis was detected only at the 20-U/ml heparin concentration and only at the first and second hours. Lymphoblasts are more sensitive to apoptotic effects of heparin than either neutrophils and mononuclear cells ($p < 0.004$). It can be suggested that low-dose heparin may cause significant apoptosis of lymphoblasts while inducing no apoptosis on neutrophils and mononuclear cells. The findings of this preliminary study indicate that further and more comprehensive research on the apoptotic effect of heparin on lymphoblasts should be done. Am. J. Hematol. 61:90–93, 1999.

Key words: apoptosis; lymphoblasts; neutrophils; mononuclear cells

INTRODUCTION

In addition to its anticoagulant activity, heparin is known to have antihypertensive [1], anti-inflammatory [2], and antiproliferative effects. All these effects are probably secondary to the existence of heparin-binding domains in a wide range of protein.

The antiproliferative effect of heparin has been studied in hepatoma cells [3] and smooth muscle cells (SMC) [4]. Heparin binds tightly to specific SMC surface receptors, accumulates within the cell, and blocks a critical point in the G_0/G_1 to S transition of the cells [4,5]. Heparin was shown to inhibit a protein kinase C-dependent pathway for cell proliferation by supressing c-fos and c-myc protooncogene expression [6] and the expression of IL-11 and GM-CSF mRNAs [7]. Recently, it was indicated that heparin induces apoptosis in human peripheral blood neutrophils [8].

The apoptotic effect of heparin may present new opportunities in the treatment of leukemia. It therefore becomes imperative to investigate the apoptotic effect of heparin. In this study, heparin-induced apoptosis on lymphoblasts in children with acute lymphoblastic leukemia (ALL), and on neutrophils and mononuclear cells in healthy children were investigated in vitro.

MATERIALS AND METHODS

Ten children aged between 3–14 years with ALL (nine had B-cell leukemia, one had T-cell leukemia) at diag-

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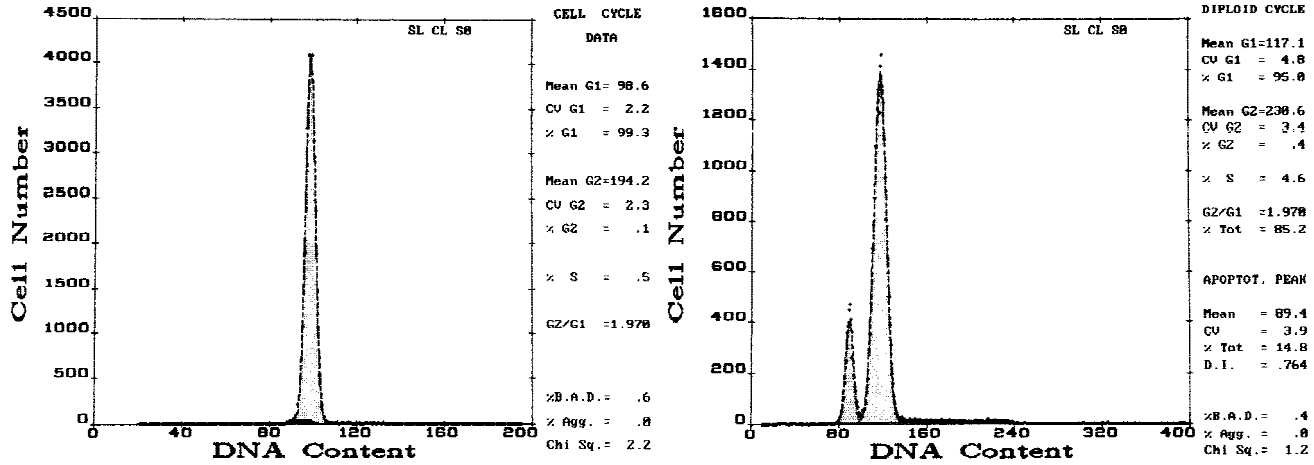

Fig. 1. The flow cytometry histograms of lymphoblasts depict DNA distribution in the 0 U/ml heparin concentration (left) and a peak less than 2n DNA (sub-G_1) in the 20 U/ml heparin concentration at the zero hour (right).

nosis (Group I), six children aged between 4–12 years with acute B-cell lymphoblastic leukemia at relapse (Group II), and 10 healthy children aged between 3–14 years (controls) were included in our study with the consent of their parents.

Separation of Blast Cells

Bone marrow samples were drawn into a tube with EDTA. Lymphoblasts were obtained by Ficoll-hypaque (Gibco BRL 13010-012) density-gradient centrifugation, washed twice with PBS. Blasts were suspended at a concentration of 2–4 × 10^5 cells/ml in RPMI 1640 medium (Sigma Diagnostica).

Separation of Neutrophils and Mononuclear Cells

Peripheral blood samples (15 ml) were drawn into a tube with EDTA. Three milliliters of Histopaque 1119 (Sigma 096H 6069) was added to a conical centrifuge tube. Three milliliters of Histopaque 1077 (Sigma 017H 6085) was overlaid on the Histopaque 1119. A 15-ml layer of blood was added with care onto the upper gradient of tubes and centrifuged at 700 g for 30 minutes at room temperature. Cells located as a layer at the upper part were mononuclear cells and those located as a layer at the lower part were neutrophils. Layers of mononuclear cells and neutrophils were aspirated with disposable plastic pipets and washed twice with PBS. Neutrophils and mononuclear cells were suspended at a concentration of 1–2 × 10^5 cells /ml in RPMI 1640 medium (Sigma-Aldrich Chemical, Gmbh, St. Louis, MO, USA).

Blast cell medium (1 ml) was placed into different heparin concentrations (0, 5, 10, 20 U/ml) in four separate tubes. The aliquots were taken following zero, one, two, and four hours and stained for DNA using the Coulter DNA-prep reagent kit (Miami, FL, USA) (CN. 6604451) according to manufacturer's recommendations. Flow cytometry was performed on a Coulter Epics Elite Flow Cytometer (Florida, USA). Data were analysed for apoptosis using the MultiCycle software. Apoptosis was measured in percentages. The same procedures were also separately performed for neutrophils and mononuclear cells.

The results were evaluated by using Wilcoxon rank sum test and Friedman two-way analysis of variance.

RESULTS

In Group I, the flow cytometry histograms of blasts displayed a peak less than 2n DNA (sub-G_1) in all different levels of heparin concentration (5, 10, 20 U/ml) at zero, one, and two hours. Such peaks are generally accepted as an indication of cells undergoing apoptosis [9]. The flow cytometry histograms of blasts depict characteristic DNA distributions in 0 U/ml and 20 U/ml heparin concentration levels at the zero hour in a patient with ALL (Group I) (Figure 1).

In Group II, similar findings were observed only for zero hour. No apoptosis was detected in blast cells without heparin in either group. Also, apoptosis was not detected at the fourth hour in either group at any heparin concentration level. A higher heparin concentration (50 U/ml) was also applied in Groups I and II. However, the samples were seen to be transformed into a gelatinous substance, hence flowcytometric analysis could not be performed.

Table I summarizes the detection of sub-G_1 peaks in blast cells for both groups. In Group I the apoptotic effect of heparin on blast cells peaked at the first hour in 5, 10, and 20 U/ml heparin levels ($p < 0.0001$). The highest apoptosis was detected in the 20 U/ml heparin level at the first hour. For Group II apoptosis was detected only at the zero hour in 5, 10, 20 U/ml heparin levels, and apoptosis was higher than that in Group I except in 5 U/ml heparin concentration ($p < 0.001$). Apoptosis was found

TABLE I. Percentage of Apoptotic Lymphoblasts at Diagnosis (Group I) and Relapse (Group II) Detected by Flow Cytometry*

		Heparin			
	Time (hour)	5 U/ml (X ± SD)	10 U/ml (X ± SD)	20 U/ml (X ± SD)	*p*
Group I	0	7.3 ± 1.1[a]	10.9 ± 1.4[d]	14.5 ± 2.5[g]	a–d < 0.004 a–g,d–g < 0.005
(*n* = 10)	1	20.1 ± 3.7[b]	24.3 ± 4.0[e]	30.1 ± 5.7[h]	b–e,e–h < 0.005 b–h < 0.02
	2	10.9 ± 1.7[c]	15.6 ± 4.6[f]	19.1 ± 2.8[i]	c–f,f–i < 0.005 c–i < 0.004
	p	a–b,b–c,a–c<0.0001	d–e,e–f,d–f<0.0001	g–h,h–i,g–i<0.0001	
Group II	0	7,1 ± 1,2[x]	14,3 ± 1,0[y]	31,1 ± 3,0[z]	x–y,x–z,y–z < 0.002
(*n* = 6)	1	—	—	—	
	2	—	—	—	
	p	a–x>0.05	d–y<0.001	g–z<0.001	

*Values are expressed as mean ± standard deviation (X ± SD).

to increase with heparin levels in Group I and Group II ($p < 0.02$, $p < 0.002$, respectively).

In the control group apoptosis was only detected in the 20 U/ml heparin concentration at the first and second hours (Table II). Apoptosis was higher at the first hour for both neutrophils and mononuclear cells than at the second hour ($p< 0.02$). Apoptosis in neutrophils both at the first and second hours was significantly higher than that in mononuclear cells ($p < 0.02$). The findings suggest that lymphoblasts are more sensitive to the apoptotic effect of heparin than both neutrophils and mononuclear cells ($p < 0.004$).

TABLE II. Percentage of Apoptotic Neutrophils and Mononuclear Cells Detected by Flow Cytometry in the 20 U/ml Heparin*

Cells	First hour (X ± SD)	Second hour (X ± SD)	*p*
Mononuclear cells (*n* = 10)	4,8 ± 0,9	2,3 ± 0,5	<0.02
Neutrophils (*n* = 10)	8,4 ± 0,7	3,8 ± 0,4	<0.02
p	<0.02	<0.02	

*Values are expressed as mean ± standard deviation (X ± SD).

DISCUSSION

Cell death involves activation of various intracellular enzymatic pathways; in necrosis this is accompanied by early membrane damage and cell disintegration. When undergoing apoptosis, cells decrease in size and their nuclei condense but they remain impermeable to vital dyes such as tryphan blue. Later, the nuclei and the cells become fragmented and cellular remnants are phagocytosed by macrophages [10]. The biochemical event that characterizes apoptosis and appears to be related to the nuclear condensation and fragmentation is double-stranded cleavage of DNA at internucleosomal sites [10,11]. The internucleosomal cutting of DNA is determined with ladder-formation on gel electrophoresis of DNA extracted from cells. This is considered diagnostic for apoptosis, but as an isolated event, and recent work has described apoptosis with no ladder formation detectable [12]. In apoptosis, flow cytometry identifies the presence of cells with low DNA stainability in a sub-G_1 DNA peak [9,13]. This low stainability usually has been interpreted as resulting from a change of DNA breakdown [9].

It was suggested that fluorescence in situ TUNEL assay, flow cytometry, and tryphan blue assay detected DNA strand breaks occurring in apoptosis and that flow cytometric detection of apoptosis was more sensitive than TUNEL assay [8]. We selected flow cytometry to look for the apoptotic effect of heparin on DNA, because TUNEL assay is a less sensitive method than flow cytometry for detection of apoptosis, and ladder-formation on gel electrophoresis of DNA extracted from cells is not considered a diagnosis for apoptosis [12].

It has been demonstrated that heparin interacts with neutrophils [14,15]. The binding of heparin to polymorphonuclear leukocytes was found to be time-dependent, saturable, specific, and reversible, and was inhibited by EDTA [15].

In this study, it was found that the low-dose heparin did not cause a high degree of apoptosis in neutrophils. Manaster et al. reported that heparin induced apoptosis in peripheral human neutrophils [8].

It should be noted that low-dose heparin caused significant levels of apoptosis in lymphoblasts, and apoptosis was found to increase with heparin levels in lympho-

blasts at diagnosis and at relapse. It could not be explained why apoptosis was higher at the zero hour in 10- and 20-U/ml heparin levels in Group II than in Group I; apoptosis was not determined at the first or second hours at any heparin level in Group II and the samples were transformed into a gelatinous substance at 50-U/ml heparin levels. It can be suggested that low-dose heparin may cause significant apoptosis of lymphoblasts while apparently not affecting neutrophils and mononuclear cells.

The findings of this preliminary study indicate that further and more comprehensive research on the apoptotic effect of heparin on lymphoblasts is needed to explore the therapeutic potential of heparin in patients with ALL.

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